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Volatiles Emitted From Eight Wound-Isolated Bacteria Differentially Attract Gravid Screwworms (Diptera: Calliphoridae) to Oviposit

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ABSTRACT Bovine blood inoculated with bacteria isolated from screwworm [*Cochliomyia hominivorax* (Coquerel) (Diptera: Calliphoridae)]-infested animal wounds was tested as an attractant for oviposition for gravid screwworms. Eight species of gram-negative coliform (Enterobacteriaceae) bacteria mixed with bovine blood singly or all species combined and incubated for various times produced volatiles that attracted gravid flies in a cage bioassay in varying numbers. In 15-min duration tests, volatiles from five species of bacteria (*Klebsiella oxytoca*, *Proteus mirabilis*, *Proteus vulgaris*, *Providencia rettgeri*, and *Providencia stuartii*) attracted more females than volatiles of the three species (*Enterobacter cloacae*, *Enterobacter sakazakii*, and *Serratia liquefaciens*). In 1-h duration oviposition tests, volatiles from the substrate using the same five species of bacteria attracted more females to oviposit than the other three species. Volatiles from 24-h incubation period elicited least attraction and oviposition whereas volatiles from the 48- and 72-h incubation period resulted in significantly more attraction and oviposition. Attraction and oviposition decreased significantly when the substrates were incubated for 96 h. Volatiles from substrate with all species of bacteria combined attracted a significantly higher percentage of flies to land and oviposit than those from substrates prepared with single species. It is possible that multiple active chemicals present in volatiles of the all-species substrate may act as synergists resulting in greater response than those observed with volatiles from single-species substrate. Before oviposition flies took a bloodmeal from the oviposition substrate. It is possible that the oviposition is moderated by two different factors in screwworm—first, by using a chemical cue to land on a potential oviposition site and second, by using a bloodmeal to stimulate oviposition.

KEY WORDS *Cochliomyia hominivorax*, attractants, bloodmeal, oviposition stimulant

The primary screwworm, *Cochliomyia hominivorax* (Coquerel) (Diptera: Calliphoridae), also known as screwworm, is an economically important pest of cattle. Larvae (maggots) feed as obligate parasites on living tissue of animals. Gravid female flies are particularly attracted to screwworm-infested wounds and the navels of newborns where the flies oviposit (Bushland 1960). Infested wounds seem to release attractive odors that act as host-finding cues. Many dipteran species are known to be attracted by host odors for feeding and oviposition. Some of these behaviors were demonstrated to be affected by the presence of bacteria. These include mosquitoes *Aedes aegypti* (L.) and *Culex pipiens molestus* (Forskal) (Ikeshoji et al. 1975); the fly *Hylemya antiqua* (Meigen) (Ikeshoji et al. 1980, Miller et al. 1984), and blow fly *Lucilia cuprina* (Wiedemann) (Emmens and Murray 1983).

Several studies on the significance of bacterial interactions with muscoid flies, such as house flies, *Musca domestica* L. (Zurek et al. 2000); stable flies, *Stomoxys calcitrans* (L.) (Lysyk et al. 1999, Romero et al. 2006); and horn flies, *Hematobia irritans* (L.) (Perotti et al. 2001), reported some symbiotic relationships during larval development and also an oviposition attractant (in stable flies). Ahmed et al. (2006) identified several species of bacteria, including *Providencia* spp. from secondary screwworm, *Cochliomyia macellaria* (F.), and showed that the development of larvae does not depend on bacteria and that some bacterial isolates negatively impact larval development. The study of the bacterial association with screwworm dates back to early seventies when DeVaney et al. (1973) reported olfactometer tests with bacteria-inoculated bovine blood showing attractancy of this material to screwworm adults. Later, Eddy et al. (1975) showed that blood inoculated and incubated with bacterium *Proteus rettgeri* attracted more adult screwworms than did three other *Proteus* species. They reported that a combination of *Bacillus* species stimulated greater oviposition from gravid females than did *Proteus* or a combination of *Bacillus* and *Proteus* species (Eddy et al. 1975). Bromel et al. 1983 isolated several species of bacteria from various stages

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of screwworm, including *Proteus mirabilis, Proteus rettgeri,* and *Providencia stuartii.* Hammack et al. (1987) showed that a steam distillate of culture medium inoculated with *Providencia* (=*Proteus*) *rettgeri* isolated from a screwworm larva strongly attracted gravid screwworm flies. Hammack (1991) reported that fresh bovine blood, which does not release the attractive odors involved in host finding, stimulated as many or more females to oviposit than did fluid from screwworm-infested wounds. Holt et al. (1979) also reported fresh bovine blood to be a contact oviposition stimulant. Gravid females also are attracted by odors from decomposing meat and liver (Bishopp 1937) and waste artificial diet from larval rearing (Adams et al. 1979; Mackley and Snow 1982; M.F.C., unpublished data). Tests conducted in large rearing cages (1 cm^3) under laboratory conditions demonstrated that bovine blood inoculated with eight species of bacteria isolated from naturally occurring screwworm-infested wounds, and incubated at 37°C for varying times, attracts gravid screwworm females to oviposit (Chaudhury et al. 2002). However, the response to individual species of bacteria was not tested, and thus, the relative importance of each of the eight species of bacteria in attracting gravid flies to oviposit had not been established.

In this article, we report the responses of gravid female screwworm flies to bovine blood inoculated with each species of bacteria separately and incubated for various times. The purpose of this investigation was to determine relative attraction as well as the rate of oviposition of the flies as influenced by the volatiles from the blood–bacteria preparation with eight species of bacteria when used as individual species or all combined. This information will allow selecting only those bacteria showing high attractiveness for additional research currently in progress in our laboratory to identify chemical(s) from the volatiles responsible for attraction and subsequent oviposition.

Materials and Methods

Insects. All tests were conducted using flies of the Panama-95 strain supplied by the Methods Development Unit of the screwworm mass-rearing facility in Tuxtla Gutierrez, Mexico as described in Chaudhury et al. (2002). Insects were reared according to the procedures described by Chaudhury and Alvarez (1999) and Chaudhury et al. (2000), and the adults were maintained at 25 ± 1°C, 50 ± 5% RH, and a photoperiod of 12:12 (L:D) h as described by Chaudhury et al. (2002). Flies were separated by sex on the day of testing by using a glass aspirator without anesthesia.

Bacteria. Bacteria were isolated from screwworm infested animal wounds (Chaudhury et al. 2002) and were identified using API-20E enteric identification system (bio-Merieux, S.A., Marcy, L'Etoile, France; bio-Merieux, Inc., Durham, NC). The species belonging to the family Enterobacteriaceae, gram-negative coliform rod-shaped bacteria were as follows: *Enterobacter cloacae* (Jordan), *Enterobacter sakazakii* Farmer, *Klebsiella oxytoca* (Flugge), *Proteus mirabilis* Hauser, *Proteus vulgaris* Hauser, *Providencia rettgeri* (Hadley, Elkins and Caldwell), *Providencia stuartii* (Buttiaux, Osteux, Fresnoy and Moriamez), and *Serratia liquefaciens* (Grimes and Hennerty).

Blood. Fresh bovine blood was collected from a local slaughter house and treated with EDTA as anticoagulant at the rate of 2 g/liter blood. Blood was refrigerated at 5°C until use on the following day.

Preparation of Blood–Bacteria Samples. Samples of each species of bacteria were grown on agar slants in culture tubes for 24 h at 37°C. Each slant culture was then transferred to 50 ml of nutrient broth (Bioxon de Mexico, Oaxaca) in 100-ml Erlenmeyer flask, which was agitated for 24 h at 37°C by using a mechanical agitator (Labline Instruments, Melrose Park, IL). One milliliter of bacterial broth from the flask was then pipetted into 49 ml of blood in an Erlenmeyer flask to make a 50-ml test sample for each of the bacteria species. The samples were incubated at 37°C for 24, 48, 72, and 96 h in separate batches and used immediately to conduct bioassay. Control samples were prepared with 49 ml of blood and 1 ml of sterile broth and were incubated and handled the same way as the treated samples. Bioassays also were conducted using blood incubated for similar times with all eight species of bacteria combined. This was prepared by combining 1 ml of each of the bacterial broth with 42 ml of blood to make a 50-ml test sample and incubating as described above. The control sample for this test was prepared with 42 ml of blood and 8 ml of sterile broth and was incubated and handled the same way as the treated samples.

Bioassay. For the attraction studies, we used the single-choice cage bioassay system described previously (Chaudhury et al. 2002). This system differs from a conventional two-choice olfactometer with directional air-flow that provides well-defined and narrow odor plumes from two (or more) sources. Smoke dispersal tests for the cage bioassay system we used indicated that the odor plume emanating from the source (at the base of the cage) becomes diffused into the entire cage in <5 min due to the normal ambient air movement under laboratory conditions. Preliminary tests before designing the experiments showed that the flies were landing almost equally in both the experimental and the control trays when both the trays were introduced in the cage simultaneously and observed for 15 min. Therefore, a sequential exposure was found more suitable for the attraction studies.

Tests were conducted according to the methods described earlier using wire mesh cages (1 by 1 by 1 m) built with a solid metal base and frame, having a 20-cm-diameter opening on the front side of the cage situated 8 cm above the base of the cage. The opening was covered and secured with a sleeve of stretch cotton-polyester material. Three cages were set up with at least a 1-m space between the cages. The room was kept well lighted during the tests by overhead fluorescent lamps (500 lux at the roof of the cage). The temperature and humidity of the room were main-

tained at 26 ± 1°C and 55 ± 5% RH, respectively. Bioassays were conducted between 0900 and 1300 hours. Cages were washed with hot water and the sleeves were replaced after each test.

One hundred 8-d-old females were introduced into each cage and were allowed 5 min to settle in the new environment. Meanwhile, each of the incubated blood samples (control or bacteria-inoculated) was poured into a plastic tray (21 by 15 cm, 3 cm in depth) lined with paper towel. The tray with the sample was then placed on the top of another plastic container (21.5 cm, 15.5 cm, 12 cm in depth) with hot water (≈60°C) to keep the blood sample warm (39–41°C) during the entire test period. Five minutes after the introduction of flies in the cage, the control sample (blood with no bacteria) was placed on the center of the bottom of the cage. Immediately after the placement of the sample, behavior of the flies was observed during a 15-min period. The number of flies remaining on the tray at the end of the 15-min period was recorded as the number attracted. The control tray was then removed from the cage taking care not to release any flies. The flies were then allowed a 10-min period to settle. After 10 min, a treated sample tray (blood with one of the bacteria species) was similarly introduced into the same cage and the observation was repeated for another 15 min and the number of flies on the tray at the end of 15-min period was recorded. We limited the observation to 15 min to avoid oviposition by the flies landed which would introduce an additional factor. Six replicates were run per incubation period, per bacteria species and six replicates for all eight species combined for each incubation period.

Oviposition Test. These tests were conducted in smaller cages (0.4 by 0.4 by 0.7 m) than the cages used for attraction bioassay as described by Chaudhury et al. (2002), using 100 8-d-old gravid females per cage. Both control and treated sample trays were introduced in the cage simultaneously and the females were given the opportunity to oviposit for 1 h under subdued light (100 lux). The sample tray was slightly modified by including a small piece of wood (0.5 by 2 by 10 cm) placed in the center of each tray that provided an oviposition substrate. After 1 h, trays were removed from the cage, the egg batches in each tray were removed, and the weight of eggs per tray was recorded. Six tests were conducted for each bacterium species incubated separately for 24-, 48-, 72-, and 96-h periods and another six tests with all eight species of bacteria together.

Dissections. Ovarian development was scored by using the scheme of Adams and Reinecke (1979). According to this scheme, females with stage 2 ovarioles possess previtellogenic oocytes in terminal follicles, whereas stage 10 ovaries exhibit terminal follicles with mature eggs that are ready to be deposited. Dissections of the alimentary tract of flies that landed or oviposited were made to record if they fed on blood upon landing on the sample tray.

Statistics. Treatment effects for landing and oviposition in terms of bacteria and incubation periods were calculated as the difference between treated and control in both experiments; response differences were evaluated with analysis of variance (ANOVA). A factorial treatment design was used followed by Tukey's (honestly significant difference [HSD]) range test to separate means after a significant F value (SAS Institute 2005).

Results

Fly Behavior. As soon as the flies were introduced in the cage, most of them settled on the ceiling and the upper part of the cage sides, with <10% resting on the base and the lower part of the sides. When the uninoculated control tray was introduced into the cage, some flight activity was observed, which subsided in 1–2 min with flies returning to resting position. Sometimes one or two flies were seen flying around the tray without landing on it. Flies that were landing on the tray normally stayed still or walked along the edges of the blood-soaked moist paper towel occasionally probing with their proboscis; these flies stayed on the surface beyond 15-min period and were counted as showing positive response. When the tray with treated sample was introduced, vigorous flight activity ensued and the flies started landing on the tray in <5 min. These flies exhibited intense probing activity along the moist edges of the paper towel and were considered showing positive attraction.

Landing Response. Results of landing response (attraction) in treated and control substrates are presented in Table 1. There was significant difference in attraction among the treatments (bacteria) ($F = 82.78$; df = 8, 24; $P < 0.001$). The mean number of flies attracted to volatiles from each of the bacteria, in general, differed from one another, with one exception being between *P. mirabilis* and *P. vulgaris.* Volatiles from *E. sakazakii* were least effective in eliciting response, whereas volatiles from *P. rettgeri* were most effective in evoking response. Ranking of the overall landing response of gravid flies to volatiles from highest to the lowest was *P. rettgeri* < *P. vulgaris* < *P. mirabilis* < *K. oxytoca* < *P. stuartii* < *S. liquefaciens* < *E. cloacae* < *E. sakazakii.* Volatiles from substrates inoculated with all eight species of bacteria attracted more flies for all incubation period. During the 15-min observation period none of the females deposited eggs. Dissection of ovaries and gut of 50% of the flies landing on the treated trays revealed stage 10 ovaries (mature eggs) for all females dissected. Except for <1% females exposed to blood with all eight species of bacteria incubated, no sign of feeding was observed.

There was also significant difference in attraction when the incubation periods were compared. The mean number flies attracted to volatiles from four different incubation periods were significantly different from one another ($F = 145.05$; df = 3, 24; $P < 0.001$). Volatiles from 24-h incubation period elicited least attraction whereas volatiles from 48- and 72-h incubation period resulted in significantly more attraction. Attraction decreased significantly when the substrates were incubated for 96 h. Although interaction between the treatments (bacteria and incubation

Table 1. Number of gravid female screwworm flies showing landing response to bovine blood inoculated with eight species of bacteria individually and collectively and incubated for 24, 48, 72, and 96 h and to corresponding controls

Bacteria	Incubation period								Mean (treated − control)[a]
	24		48		72		96		
	Treated	Control	Treated	Control	Treated	Control	Treated	Control	
Enterobacter cloacae	0	0	3.7	0	5.3	0	1.3	0	2.58ef
Enterobacter sakazakii	0	0	1.8	0	2	0	1.2	0	1.25f
Klebsiella oxytoca	1.2	0	5.7	0.8	10.3	0.8	3.5	0	4.75cd
Proteus mirabilis	0.8	0	7.5	0.6	11.7	0.3	2.7	0	5.42c
Proteus vulgaris	2.8	0	10	0.5	10.2	0.2	4.5	0	6.71c
Providencia rettgeri	3.7	0.5	11.2	0.7	13.3	1.3	5	0.5	7.58b
Providencia stuartii	1.3	0	6	0.5	9.8	0.5	3	0.5	4.71cd
Serratia liquefaciens	0.3	0	4	0	7.7	0.8	2.2	0	3.33de
All species	7.7	2	26.7	1.8	30.2	2	10	1	16.92a
Mean (treated − control)[b]	1.7d		7.81b		10.43a		3.3c		

[a] Mean (for each treatment for all periods) followed by the same letter are not significantly different ($P = 0.05$; Tukey's range test).
[b] Mean (for each incubation period for all treatments) followed by the same letter are not significantly different ($P = 0.05$; Tukey's range test).

time) was significant, graphic evaluation of the means (not presented) showed the treatments responded in similar patterns but the magnitude of response from the combined bacteria treatment was much greater; therefore, the interaction did not impact the treatment comparisons/interpretations.

Oviposition. The amount of eggs deposited by 8-d-old gravid females during 1-h period on treated substrates with single species and eight species combined bacteria and corresponding controls (uninoculated substrates) are shown in Table 2. For the single-species tests, eggs were deposited in all bacteria-inoculated substrates incubated for 48-, 72-, and 96-h periods, and only on substrates inoculated with *P. mirabilis* and *P. rettgeri* that were incubated for 24 h. No eggs were deposited in most of the control experiments with 24- and 96-h incubation. An analysis of the difference between the amount of eggs from treated and control substrates showed that the amount of eggs from bacteria-inoculated substrates were significantly different from one another for some of the treatments ($F = 341.07$; df = 8, 24; $P < 0.001$) (Table 2). In the single-species tests, the highest amount of eggs was recorded from *P. vulgaris* and *P. rettgeri* inoculated substrates, and the least from *E. cloacae* and *E. sakazakii* inoculated substrates. Significantly more eggs were deposited in the substrates inoculated with all eight species of bacteria. Amount of eggs laid within the four incubation periods were significantly different from one another ($F = 206.91$; df = 3, 24; $P < 0.001$). Oviposition was lowest on the substrates with 24-h incubation and increased significantly with the increase of incubation period to 48 and 72 h; however, there was a significant decrease in egg laying when 96-h incubated blood was offered (Table 2).

Dissections of the gut and ovary of 20 females immediately after the completion of each oviposition test revealed sign of bloodmeal in the gut of those females which deposited their eggs, except <1% of total flies dissected for each treatment did not oviposit in spite of having a bloodmeal. It was not determined whether the bloodmeal was from the inoculated substrate or from the control.

Discussion

Results of our cage bioassays using substrate inoculated with single species showed that all eight species

Table 2. Amount of eggs (milligrams) deposited by screwworm flies during 1-h period on the substrate with blood inoculated with indicated bacteria (treated), and the respective control (without bacteria) and incubated for indicated time periods

Bacteria	Incubation period								Mean (treated − control)[a]
	24		48		72		96		
	Treated	Control	Treated	Control	Treated	Control	Treated	Control	
Enterobacter cloacae	0	0	18	0	44	6	36	0	3.83d
Enterobacter sakazakii	0	0	45	0	64	0	28	0	5.71d
Klebsiella oxytoca	0	0	478	70	522	32	67	15	39.54b
Proteus mirabilis	53	0	383	45	488	46	41	0	35.92b
Proteus vulgaris	0	0	612	28	586	25	121	24	51.75b
Providencia rettgeri	33	0	231	34	605	38	233	0	51.71b
Providencia stuartii	0	0	239	0	507	41	36	0	30.88bc
Serratia liquefaciens	0	0	66	0	195	15	25	0	11.29cd
All species	45	0	2528	164	3075	181	1705	93	288a
Mean (treated − control)[b]	2.24d		82.78b		105.54a		39.94c		

[a] Mean (for each treatment for all periods) followed by the same letter are not significantly different ($P = 0.05$; Tukey's range test).
[b] Mean (for each incubation period for all treatments) followed by the same letter are not significantly different ($P = 0.05$; Tukey's range test).

of bacteria tested produced volatiles which attracted gravid females to land on inoculated bovine blood substrate during 15-min test period and deposited eggs during 1-h test period. The control substrates elicited lower or no response, suggesting that no other factors played a significant role in eliciting attraction. The extent of attraction and oviposition varied according to the bacteria species and the length of the incubation period. These results suggest that not all species of bacteria produced the same effective attractants at the same rate. It is likely that the composition of the resulting volatiles varied from species to species qualitatively, such as the absence/presence of specific effective chemical(s) or quantitatively, such as the number of effective chemical molecules available to elicit the behavior, or both. Relative volatility of the effective chemical (s) also may have influenced the fly behavior. Results indicate that *P. mirabilis*, *P. vulgaris*, *Providencia rettgeri*, *P. stuartii*, and *K. oxytoca* produced relatively more attractive factors than the two *Enterobacter* spp. and *S. liquefaciens.* Other workers have reported the attractive quality of only *P. rettgeri* (DeVaney et al. 1973, Eddy et al. 1975, Hammack et al. 1987). These authors found *Providencia rettgeri* (reported as *Proteus rettgeri*) cultures to be attractive in olfactometer tests (*Providencia rettgeri* and *Proteus rettgeri* are considered homotypic synonyms and share the same type strain; Skerman et al. 1980). Although *Enterobacter*, *Klebsiella*, and *Serratia* spp. were reported earlier from various stages of screwworm, these species were never tested for their effectiveness to produce attractive volatiles for screwworm oviposition (Bromel et al. 1983). Interestingly, Bromel et al. (1983) reported a *Serratia* sp. isolated from screwworm eggs; this suggests a possible relationship with oviposition. It is important to emphasize that all the bacteria used in our studies were isolated from naturally occurring screwworm-infested animal wounds where as the bacteria used by previous authors were either originated from various stages of screwworm or a combination of bacteria purchased from commercial depository, species isolated from larval media or wound fluid. We reported results from eight species of bacteria isolated only from the infested animal wounds including new results from three species (*E. cloacae*, *E. sakazakii*, and *K. oxytoca*), which was not reported by earlier authors. Sometimes, some of the previous workers tested bacteria from combined sources making it difficult to interpret some of the results (DeVaney et al. 1973). Eddy et al. (1975) also tested *Proteus morganii* and 12 *Bacillus* spp., which showed varying degrees of attractiveness and oviposition. These species were not isolated from screwworm-infested wounds in our studies (Chaudhury et al. 2002). Previous authors conducted their oviposition tests in closed shell vials (Eddy et al. 1975). We conducted the oviposition tests in cages to assess both attraction from a distance and oviposition on contact. Furthermore, we used wooden pieces as oviposition substrate instead of cotton wool used by other workers (Hammack 1991). Pieces of wood are routinely used for the screwworm rearing program because of the ease and simplicity of removing eggs from wooden surfaces for measurement (Chaudhury et al. 2002; M.F.C., unpublished data). Results of these studies will be useful in optimizing oviposition in cages of the rearing colony.

The substrates prepared and tested with all species combined elicited landing response that was higher than any of those elicited by the single species, indicating a possible cumulative effect of the total volatiles produced, although the percent response of all species together was less than the cumulative total percent response for all the single species. It is possible that multiple active chemicals present in the volatiles of all-species substrate may act as synergists resulting in greater response than those observed with volatiles from single-species substrate.

Incubation period of the inoculated substrate seems to be critical. A detailed examination of this factor has not been done by earlier authors. In our study, an incubation period of 24 h was not sufficient to elicit significant attraction or oviposition. Both attraction and oviposition were improved when the substrate was incubated for 48 and 72 h. It is possible that the length of incubation time affects the amount of attractive volatiles produced. It is also possible that fresh blood contains inhibitors that are broken down over time by incubation with bacteria. An incubation period of 96 h resulted in decrease in attraction as well as oviposition. It is possible that prolonged incubation resulted in production of antagonist metabolic compound(s), masking properties of attractant chemicals; this also could be due to high mortality of bacteria resulting from an increase in population during the long incubation period causing depletion of nutrients.

Results of the oviposition tests and the dissection showing presence of blood in the guts of the dissected females which oviposited indicate that the bloodmeal may play a role in stimulating oviposition. A few flies oviposited in the control (uninoculated) substrates (Table 2), which suggests that an uninoculated bloodmeal is also able to stimulate oviposition. Feeding of blood by ovipositing females was reported by other workers (Holt et al. 1979, Hammack 1991). Alternatively, dissections revealed that a few flies (<1%) had bloodmeal but did not deposit their eggs. However, it was not known whether they had the bloodmeal from the treated or the control substrate. It is likely that chemicals from volatiles originating from bacteria-inoculated blood are responsible for attracting the gravid flies from a distance to the potential oviposition substrate; once the flies are in contact with the substrate, they are stimulated to feed; then feeding stimulates oviposition. Holt et al. (1979) and Hammack (1991) reported feeding of flies accompanied oviposition and suggested that chemical stimuli, other than an attractant, may be important to stimulate actual oviposition.

These results indicate that it may be necessary to use all the bacteria species in a substrate and to incubate the substrate at least for 72 h to obtain the most effective volatiles. Protocols from these results are being considered in our laboratory for collection of

bacteria-produced volatiles using solid phase microextraction system and identification of effective chemicals from these volatiles. Additional work will be needed to determine optimum aliquot size of each bacteria species in the substrate (bacteria population size) to produce maximum attraction.

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